EXHIBIT E

PATENT
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IN THE UNITED STATES PATENT
AND TRADEMARK OFFICE

Applicants: Alitalo et al.

Serial No.: 09/169,079

Filed: October 9, 1998

For: Flt4 (VEGFR-3) as a Target for Tumor Imaging and Anti-Tumor Therapy

Examiner: Joseph F. Murphy

Group Art Unit: 1646

I hereby certify that this paper is being deposited with the United States Postal Service as first class mail, postage prepaid, in an envelope addressed to: Assistant Commissioner for Patents, Washington, DC 20231 on this date:

May 10, 2002

Eric M. Brusca

Declaration Pursuant to 37 C.F.R. § 1.132 of Kari Alitalo

I, Dr. Kari Alitalo, declare and state as follows:

Introduction

I am a co-inventor of subject matter of the above-identified patent application (hereinafter "the patent application"). I make this declaration to provide evidence to the Patent Office that may be relevant to the patentability of pending claims in the patent application that relate to methods of treatment. Specifically, I understand from my attorneys that the Patent Office has reviewed the patent application and has alleged that the application does not adequately teach how to effectively treat any disease or achieve a therapeutic benefit in humans by administering a composition comprising a compound to inhibit Flt4 binding to its ligand(s).

In a first declaration executed on January 31, 2001, I described experiments and data that provided evidence of therapeutic efficacy in vivo for the methods described and claimed in the patent application.

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In this second declaration, my attorneys have asked me to describe recent data that is relavent to the therapeutic potential of Flt4 inhibitors in tumor therapy. Specifically, I have been asked to describe recent research in my lab with regard to the impact of soluble Flt4 molecules on inhibiting lymphangiogenesis and lymphatic metastasis. I have also been asked to elaborate on the differential expression levels observed between cancerous versus healthy human tissues and between developing embryos versus adult tissues.

Evidence

Since the patent application was filed, my laboratory has obtained additional research results that evince the therapeutic efficacy of the methods of treatment described and claimed in the patent application. I summarize the protocols and data below, which show that inhibition of the Flt4–VEGF-C interaction suppresses tumor lymphangiogenesis and lymph node metastasis.

A. OVERVIEW

A human lung cancer cell line (NCI-H460-LNM35) with a high frequency of lymphatic metastasis was chosen to investigate the efficacy of soluble Flt4 as an inhibitor of tumor metastasis (Kozaki et al., Cancer Res, 60:2535-40(2000)). VEGF-C production was found to be considerably increased in these cells when compared with the parental cell line (NCI-H460-N15), which has a low metastatic capacity. LNM35 tumors, unlike N15 tumors, were lymphangiogenic and axillary lymph nodes from mice carrying the LNM35 tumors were significantly enlarged and almost completely occupied by the tumor cells. However, tumor lymphangiogenesis and lymph node metastases were inhibited in LNM35 tumors engineered to secrete a soluble form of VEGFR-3 (VEGFR-3-Ig). Essentially no lymph node metastases were detected in mice treated with a recombinant adenovirus expressing the VEGFR-3-Ig fusion protein. These results indicate that inhibition of growth factor signalling via VEGFR-3 can suppress tumor lymphangiogenesis and metastasis to regional lymph nodes.

B. MATERIALS AND METHODS

1. Cell lines and transfections

The human lung cancer cell line NCI-H460-LNM35 is a subline of NCI-H460-N15, a human large cell carcinoma of the lung, established as previously

described [Kozaki et al., Cancer Res, 60:2535-40, 2000; Kozaki et al., Oncogene, 20:4228-34, 2001]. LNM35 cells and their parental N15 cells were maintained in RPMI 1640 medium supplemented with 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 µg/ml), and 10% fetal bovine serum (Gibco BRL). LNM35 cells were transfected with the pEBS7/VEGFR-3-Ig [Karpanen et al., Cancer Res, 61:1786-90, 2001.] or with pEBS7 vector, and N15 cells with the pEBS7/VEGF-C [Karpanen et al., Cancer Res, 61:1786-90, 2001.] or with pEBS7 by using liposomes (FuGENE 6, Roche). Vector pEBS7 is an Epstein-Barr virus-based vector that yields high-frequency transformation in certain human cell lines (Peterson et al., Gene(Amst.), 107:279-284, 1991). VEGFR-3-Ig, containing the first three immunoglobulin homology domains of VEGFR-3 fused to the Fc domain of human immunoblobulin _ chain, and VEGF-C were introduced into the pEBS7 expression vector and transfected into the specified cell lines. The transfected cells were then selected in hygromycin (400 µg/ml, Calbiochem), until resistant cell pools were obtained.

2. Xenotransplantation and analysis of tumors

Approximately 1.0×10^7 LNM35/VEGFR-3-Ig, LNM35/pEBS7, N15/VEGF-C or N15/pEBS7 cells in 100 µl of serum-free medium were implanted in the subcutaneous tissue of the right abdominal wall of female SCID mice (7 to 8-week-old). The tumors were measured with digital calipers and the tumor volumes were calculated as follows: volume = length $\times$ width$^2 \times 0.52$ (mm^3). Mice were sacrificed after 6 weeks, and the tumors and some internal organs including the lungs as well as axillary lymph nodes were collected. The length and width of lymph nodes were also measured and statistical analysis was performed using the unpaired T test. Samples of each tumor were either snap-frozen in liquid nitrogen and stored at -70 °C for protein analysis or fixed immediately in 4% paraformaldehyde overnight at 4 °C and then processed for further histological analysis.

In separate experiments, approximately 1.0×10^7 LNM35 cells were also subcutaneously implanted into SCID mice. Recombinant adenoviruses expressing the VEGFR-3-Ig fusion protein (AdR3-Ig) [Karpanen et al., Cancer Res, 61:1786-90, 2001.] or ß-galactosidase (AdLacZ) [Laitinen et al., Hum Gene Ther, 9:1481-6, 1998] (10^9 pfu) were administered via the tail vein on the day of tumor implantation. Blood from the treated and control mice was collected and the serum

concentration of VEGFR-3-Ig was determined by ELISA [Makinen et al., Nat Med, 7:199-205, 2001]. Mice were sacrificed five weeks later, and their tissues were collected and processed as above.

3. Histology

Paraffin sections of tumors (6 μm) were immunostained with monoclonal antibodies against PECAM-1 (PharMingen), which is typically an antigen that is shared by both endothelial and distinct hematopoietic cells, polyclonal antibodies against LYVE-1(lymphatic vessel endothelial HA receptor) [Prevo et al., J Biol Chem, 276:19420-30, 2001], a HA receptor expressed predominantly in lymphatic vessels, or Biotin-mouse anti-human IgG1 antibodies (Zymed). Sections of the lungs and axillary lymph nodes were stained with haematoxylin and eosin.

4. Immunoprecipitation and western blotting analysis

The cells were metabolically labeled in methionine and cysteine free MEM (Life Technologies, Inc.) supplemented with 100 μCi/ml of [^{35}S]Met/[^{35}S]Cys (Promix, Amersham Pharmacia Biotech) for about 8 h. The conditioned medium was then harvested and cleared by centrifugation. VEGF-C was immnoprecipitated from the medium by using polyclonal antibodies against VEGF-C [Joukov et al., Embo J, 16:3898-911, 1997] followed by incubation with protein A-sepharose (Amersham Pharmacia Biotech). The complexes were then washed twice with 0.5% BSA/0.02% Tween 20 in PBS, and once with PBS, and analysed by SDS-PAGE under reducing conditions.

Conditioned media from the cell cultures and tumor samples were analysed for expression of the soluble VEGFR-3-Ig fusion protein. Frozen tumor samples were homogenized on ice in the lysis buffer (20 mM Tris, pH 7.6, 1 mM EDTA, 50 mM NaCl, 50 mM NaF, 1% Triton-X100, supplemented with 1 mM PMSF, 1 mU/ml approtinin, 1 mM Na_3VO_4 and 10 μg/ml leupeptin). The fusion protein was bound to protein A sepharose (Pharmacia Biotech) from the LNM35/VEGFR-3-Ig cell conditioned medium, or from the tumor lysates containing equal amounts of total protein (1.5 mg) as determined by the BCA protein assay (Pierce, Rockford, Illinois). The bound proteins were dissolved in reducing SDS-PAGE sample buffer, separated in 8% SDS-PAGE and transferred to nitrocellulose. The blots were probed with horseradish peroxidase conjugated goat-anti-human IgG

(Fc) antibody (KPL) and developed using the enhanced chemiluminescence detection system.

5. Analysis of RNA

Extraction of RNA from LNM35 and N15 cells, northern blotting and hybridization analysis was performed according to standard procedures. cDNA probes were generated by PCR using the following primers: VEGF sense primer: AGC TCT AGA CAT CAC GAA GTG GTG AAG TT, antisense primer: AGC GAA TTC TTG TCA CAT CTG CAA GTA CG; VEGF-B sense primer: AGC TCT AGA AGA TGT GTA TAC TCG CGC TA, antisense primer: AGC GAA TTC CCT TGG CAA CGG AGG AAG CT; VEGF-C sense primer: AGC TCT AGA CCA GTG TAG ATG AAC TCA TG, antisense primer: AGC GAA TTC AGC CAG GCA TCT GCA GAT GT; VEGF-D sense primer: AGC TCT AGA TGT AAG TGC TTG CCA ACA GC, antisense primer: AGC GAA TTC AGC GGC AAT GCT TTG CAC AT.

C. RESULTS

1. Analysis of VEGF-C and VEGF-D expression

LNM35 is a human large cell carcinoma of the lung, which shows consistent lymphogenous metastasis, while its parental cell line N15 does not have this property [Kozaki et al., Cancer Res, 60:2535-40, 2000]. We examined expression of the VEGFs in these cells. Northern blotting and hybridization analysis demonstrated that VEGF and VEGF-B were equally expressed by both cell lines, but VEGF-C mRNA was considerably increased in LNM35 cells in comparison with N15 cells. No expression of VEGF-D mRNA could be detected. The enhanced secretion of the VEGF-C protein was confirmed by immunoprecipitation using polyclonal antibodies against VEGF-C. We also confirmed that the N15 cells transfected with the VEGF-C expression vector secrete abundant amounts of VEGF-C into the culture medium.

2. Establishment of LNM35 cells overexpressing the soluble VEGFR-3

For studies of the effect of soluble VEGFR-3-Ig expression on tumor lymphangiogenesis, LNM35 cells were transfected with the pEBS7/VEGFR-3-Ig expression vector and pools of hygromycin selected cell clones were obtained. The pools were characterized for protein secretion by metabolic labeling and

immunoprecipitation as well as western blotting analysis. The serum-free medium conditioned by these cells contained the VEGFR-3-Ig fusion protein which was absent from the LNM35 cells transfected with pEBS7 vector (Fig. 1C). Both cell pools were microscopically indistinguishable and had similar growth rates in vitro.

3. Persistent expression of VEGFR-3-Ig in the tumors

LNM35/pEBS7 and LNM35/pEBS7/VEGFR-3-Ig cells were subcutaneously injected into the right abdominal wall of SCID mice. The tumors formed by the two cell lines *in vivo* had similar growth rates. The tumors were excised six weeks after implantation and tumor lysates were analysed by western blotting using antibodies against human immunoglobulin gamma Fc part (IgG). This showed expression of VEGFR-3-Ig in the LNM35/VEGFR-3-Ig tumors. Expression of the VEGFR-3-Ig protein was also confirmed by immunostaining of the tumors and of the lung metastases. No staining was obtained in vector-transfected tumors or metastases.

4. Inhibition of tumor lymphangiogenesis by the soluble VEGFR-3

The occurrence of lymphatic vessels in the tumors was analysed by immunostaining using antibodies against the lymphatic endothelial marker LYVE-1 [Prevo et al., J Biol Chem, 276:19420-30, 2001; 33]. The staining of sections from the control tumors showed that LYVE-1 positive vessel-like structures were formed within the LNM35 tumors. Such structures were often observed as clusters of vessels in some regions of the tumor. Hyperplastic lymphatic vessels filled with tumor cells were also observed in the tumor periphery. In contrast, few LYVE-1 positive vessel-like structures were observed in the sections from the VEGFR-3-Ig tumors. The average number of LYVE-1 positive vessels was determined from three microscopic fields of the highest vessel density (VEGFR-3-Ig: 4.1 ± 0.6 vessels/grid; Control: 15.3 ± 1.7; the two tailed P value equals 0.0001). Staining of tumor sections for PECAM-1, a panendothelial marker, showed that there was also a significant difference in vessel density between the control LNM35 tumors and the transfected derivatives secreting the soluble VEGFR-3-Ig (VEGFR-3-Ig: 17.1 ± 3.7 vessels/grid; Control: 23.9 ± 4.9; P=0.0283). The small trend towards higher PECAM-1 vessel counts and the occurrence of larger vessels in the vector-transfected tumors may depend on the fact that the lymphatic vessels were also stained by the anti-PECAM-1 antibodies.

Inhibition of tumor lymphangiogenesis was also observed in mice treated with AdR3-Ig.

In the N15 tumors, much less lymphangiogenesis occurred in comparison with the LNM35 tumors. However, hyperplastic lymphatic vessels were evident between the expanding tumor foci in the N15/VEGF-C tumors.

5. Suppression of axillary lymph node metastasis

Statistical analysis showed a significant difference in lymph node size between a mouse carrying the VEGFR-3-Ig producing tumor and from a mouse carrying the control LNM35 tumor. In the VEGFR-3-Ig group the lymph node length was 2.45 ± 1.52 mm and width 1.93 ± 1.24 mm; while in the control group these parameters were 4.92 ± 2.24 mm and 3.79 ± 1.93 mm (mean ± SD, n=12) respectively. These differences were highly significant (P=0.0045 and 0.0102 respectively). Histological analysis revealed that 12 out of 12 lymph nodes from the control group but only 4 out of 12 lymph nodes from the VEGFR-3-Ig group contained metastases.

In the LNM35 tumor-bearing mice treated with AdR3-Ig, high concentrations of the VEGFR3-Ig fusion protein were detected in the circulation. Six of seven mice receiving AdLacZ in the control group showed macroscopic lymph node metastasis, and 11 out of 14 axillary lymph nodes were confirmed to have metastasis by histological analysis. However, no macroscopic or microscopic metastases were observed in 14 mice treated with AdR3-Ig. Again statistical analysis showed a very significant difference in lymph node size between the two groups (P<0.0001). In the AdR3-Ig treated group the lymph node length was 2.11 ± 0.23 mm and width 1.56 ± 0.13 mm, n=14; while in the control group these parameters were 5.24 ± 1.84 mm and 4.3 ± 1.64 mm (mean ± SD, n=7) respectively.

There was no difference in lymph node size observed between the N15/VEGF-C and N15/pEBS7 groups (data not shown), and also no increase of lymph node metastasis was detected in mice bearing the N15/VEGF-C tumors.

6. Lung metastasis

Numerous metastatic tumor nodules were observed in the lungs from both the VEGFR-3-Ig expressing and control groups. Lung metastasis was also

observed in both AdR3-Ig treated and control mice. No significant difference was observed in the number of tumor nodules between the two groups. There were always more tumor nodules visible on the anterior side of the lungs than on the posterior side. Histological analysis revealed that the metastases invaded the lung tissue and did not grow just by expansion.

D. ANALYSIS

This study demonstrates that inhibition of VEGFR-3 signalling can suppress tumor lymphangiogenesis and lymphatic metastasis. However, such inhibition did not apply to lung metastases, suggesting that the mechanisms of lymphatic and lung metastasis differ at least in the present model, which involve a cell line derived from the lung. According to our results, VEGF-C overexpression and the associated de novo formation of lymphatic vessels are necessary but not sufficient for the metastatic dissemination of tumor cells to the lymph nodes. Apparently additional factors besides VEGF-C are thus required for metastatic spread.

LNM35 cells are capable of spontaneous metastasis to regional lymph nodes and systemically to the lungs at 100% incidence even when subcutaneously implanted [Kozaki et al., Cancer Res, 60:2535-40, 2000]. As the VEGF family members play important roles in angiogenesis and/or lymphangiogenesis and have been shown to be required for tumor growth and metastasis, we compared expression of the VEGF family members in the highly metastatic LNM35 cells and in the parental N15 cells. Northern blotting and immunoprecipitation analysis demonstrated that VEGF-C expression was significantly enhanced in LNM35 cells in comparison with N15 cells, while expression of VEGF-D was not detected in either cell line. There was also no significant difference in VEGF or VEGF-B expression between the two cell lines. Consistent with this observation, LYVE-1 positive vessel-like structures were present at a much higher density in the LNM35 tumors than in the N15 tumors. These findings agree with the recently published direct evidence for the role of VEGF-C in tumor lymphangiogenesis and tumor cell dissemination [Mandriota et al., Embo J, 20:672-82, 2001; Skobe et al., Nat Med, 7:192-8, 2001; Karpanen et al., Cancer Res, 61:1786-90, 2001.]. Therefore enhanced VEGF-C expression may at least partly account for the invasiveness of LNM35 cells. However, overexpression of VEGF-C in the low metastatic N15 cells was not sufficient to

convert these cells into a high metastatic phenotype, suggesting that additional factors are involved in the control of the metastatic phenotype.

To investigate the consequences of blocking VEGFR-3 signalling in a metastatic tumor, two stable tumor cell lines were generated, one of which expressed VEGFR-3-Ig and another one transfected with the vector plasmid as a control. Western blotting and immunostaining analysis showed that expression of the soluble receptor persisted in the tumors during the study. Hyperplastic lymphatic vessels filled with tumor cells were observed in the periphery of the control LNM35 tumors. These resembled the lymphatic vessels previously observed in VEGF-C overexpressing tumors [Karpanen et al., Cancer Res, 61:1786-90, 2001.]. In addition, lymphatic vessel clusters with open lumens were present within the LNM35 tumors. However, the lymphatic vessels were not evenly distributed within the tumor, which may reflect variation of the local tumor microenvironment, such as differences in growth factor concentrations. In clinical cancer intratumoral lymphatics are rare [de Waal et al., Am J Pathol, 150:1951-7, 1997], and it has been proposed that they collapse within the tumor due to mechanical stress generated by proliferating cancer cells and high interstitial pressure due to plasma leakage [Pepper et al., Clin Cancer Res, 7:462-8, 2001; Leu et al., Cancer Res, 60:4324-7, 2000]. At least some of the intratumoral lymphatic vessels in the control LNM35 tumors may result from trapping of peritumoral lymphatic vessels in between the growing tumor foci. In the present study, inhibition of tumor-associated lymphatic vessels was obtained by overexpression of the soluble VEGFR-3-Ig fusion protein in the tumor cells, and also by adenoviral expression of this protein in the liver. This result is consistent with the inhibition of peritumoral lymphatic vessels in VEGF-C-transfected MCF-7 breast carcinoma xenografts of SCID mice infected with the recombinant adenovirus [Karpanen et al., Cancer Res, 61:1786-90, 2001; see also first Alitalo Declaration] .

Only lymphatic vessels were stained by anti-VEGFR-3 antibodies in the skin and in peritumoral areas. Within the tumors, both lymphatic and blood vessels were VEGFR-3-positive. It has been suggested that VEGFR-3 induction in endothelial cells of tumor blood vessels plays a role in tumor angiogenesis [Leu et al., Cancer Res, 60:4324-7, 2000; Partanen et al., Cancer, 86:2406-12, 1999; Valtola et al., Am J Pathol, 154:1381-90, 1999; Kubo et al., Blood, 96:546-53, 2000]. However, in the present study the soluble VEGFR-3 fusion protein overexpressed in

the tumor cells did not affect the tumor growth rate, and there was also no significant difference in the density of intratumoral blood vessels. This agrees with the previous result that a soluble form of VEGFR-3 did not inhibit tumor angiogenesis or growth [Siemeister et al., Cancer Res, 59:3185-91, 1999]. Thus the data suggests that VEGFR-3 expressed in the endothelial cells of tumor blood vessels may have a redundant function in tumor angiogenesis, at least in some tumors, and that targeting VEGFR-3 should be part of a multi-pronged approach to tumor therapy.

Tumor metastasis to regional lymph nodes is common in many types of human cancers and a correlation between lymphangiogenesis and tumor metastasis has been shown recently. Our study extends these findings by showing that inhibition of lymphangiogenesis suppresses lymph node metastasis. Histological analysis demonstrated that most axillary lymph nodes of mice bearing the control LNM35 tumors were almost completely occupied by the tumor cells. In contrast, there was only one lymph node showing macroscopic evidence of metastasis in mice bearing the VEGFR-3-Ig expressing tumors. A similar observation was also made in separate experiments where a recombinant adenovirus expressing the VEGFR-3-Ig fusion protein was applied via the tail vein while LNM35 cells were implanted subcutaneously. Strikingly no macroscopic or microscopic metastases were observed in the axillary lymph nodes of the AdR3-Ig treated mice by five weeks after tumor implantation. However, macroscopically evident lymph node metastases were seen in the control mice injected with AdLacZ. Yet lung metastases occurred in all these mice. This suggests that LNM35 tumor cells, which are derived from the lung itself, can use other mechanisms or routes for metastasis to the lung, for example via the bloodstream. The infiltration of LNM35 tumor cells into blood vessels has been described previously [Kozaki et al., Cancer Res, 60:2535-40, 2000] and was also observed in this study. Given the results described above and previously, treatments targeted to Flt4 have the potential to prevent the further dissemination of tumor cells to other organs through lymphatic routes, a common mode of dissemination of tumor cells throughout the body. As such, the data shows that anti-Flt4 therapy should form part of a multi-drug therapies to target lymphatics in combination with other anti-cancer medicaments in the treatment of a variety of cancers.

Tumor cell spread to local lymph nodes is an early event during tumor metastasis. Results of our study provide direct evidence for the hypothesis that

inhibition of VEGFR-3 signalling can block tumor lymphangiogenesis and suppress lymph node metastasis. However, in our cells, VEGF-C was not the only metastasis rate-limiting factor. Furthermore, evidence was obtained that the inhibition of visceral metastasis requires the blocking of other routes such as tumor spread via blood vessels.

Certification

I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code, and that such willful, false statements may jeopardize the validity of the application or any patent issued thereon.

Kari Alitalo

Date: May 1, 2002